

U.S. Patent Application No. 10/524,278  
Amendment After Final dated April 14, 2008  
Reply to Final Office Action of January 15, 2008

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**REMARKS/ARGUMENTS**

Reconsideration and continued examination of the above-identified application are respectfully requested.

By way of this amendment, claims 1, 5, 6, 13, and 20 have been amended. Claim 22 has been canceled. Full support for the amendment can be found throughout the present application and the claims as originally filed, particularly, at pages 2, 5, 7-8, and 13 of the present application. Therefore, no new questions of patentability should arise nor does the amendment necessitate any further searching on the part of the Examiner. The amendment places the application in condition for allowance. At a minimum, the amendment places the application in a better condition for appeal. Accordingly, no questions of new matter should arise and entry of the amendment is respectfully requested.

**Rejection of Claims 1-2, 5-6, 10, 13, and 19-22 under 35 U.S.C. §112 – First Paragraph**

Beginning at page 4 of the Office Action and continuing to page 9, the Examiner maintains the rejection of claims 1-2, 5-6, 10, 13, and 19-22 under 35 U.S.C. §112, first paragraph, as failing to comply with the enablement requirement. The Examiner states that the claims contain subject matter which was not described in the specification in such a way as to enable one skilled in the art to make and/or use the invention. The Examiner essentially restates the rejection presented in the previous Office Action. The Examiner states that it is unpredictable whether isoforms of UGT would have similar enzyme activity with respect to drug glucuronidation. The Examiner states that the specification does not analyze UGT1A1 and UGT1A6 with respect to the same substrate. The Examiner also states that Kurkela does not support Applicants' assertion that drug metabolism can be predicted for the class of drugs

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encompassed by the claims once a mutation in Y483D of UGT1A9, corresponding to Y486D of UGT1A1, is detected. The Examiner concludes, therefore, that one cannot predict the effects that a mutation in different isoforms of UGT will have with respect to glucuronidation of various drugs. This rejection is respectfully traversed.

The present specification does provide adequate enablement for the present claims. In order to assist the Examiner, however, the claims have been amended to further define the invention. Claims 1 and 20 recite, in part, an assay method for drug glucuronidation of UDP-glucuronosyltransferase 1 (UGT1) of a subject, comprising detecting a mutation(s) in an exon 5 region of a gene coding for UGT1 and determining that, when the mutation in an exon 5 region encoding a UGT1 molecule is detected, the level of enzymatic activity of the UGT1 molecule in drug glucuronidation in the subject would be different than that of a UGT1 molecule without the mutation, wherein the mutation is limited to mutation at the nucleotide position corresponding to position 1456 of UGT1A1, the drug is limited 2-amino-5-nitro-4-trifluoromethylphenol, and the subject is limited to humans. Claim 13 has been amended to further recite that the detection device can be used to detect a mutation in an exon 5 region of a gene coding for UGT1 that correspond(s) to nucleotide number 1456 in the genetic sequence of UGT1 which encodes an amino acid at position 486 in the amino acid sequence of UGT1A1 molecule and that the method comprises determining that, when the mutation in an exon 5 region encoding a UGT1 molecule is detected, the level of enzymatic activity of the UGT1 molecule in glucuronidation of 2-amino-5-nitro-4-trifluoromethylphenol in the subject would be different than that of a UGT1 molecule without the mutation. Thus, the "mutation" recited in the claims is limited to mutation at the nucleotide position corresponding to position 1456 of UGT1A1, the "drug" recited in the claims is limited to 2-amino-5-nitro-4-trifluoromethylphenol, and the subject recited in the claims is

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limited to humans. Accordingly, this aspect of the rejection should be withdrawn.

Further, it should be noted that by assaying a mutation of nucleotide number 1456 in the exon 5 region of UGT1A1 and mutations in positions corresponding to nucleotide number 1456 in other isoforms of UGT1, it is possible to predict drug metabolism (Present application, page 5). As described in the present application, UGTs are grouped into two families, UGT1 and UGT2 (Present application, page 1). UGT1 and UGT2 are different genes and that UGT1 isoforms, UGT1A1, UGT1A2, UGT1A3, UGT1A4, UGT1A5, UGT1A6, UGT1A7, UGT1A8, UGT1A9, and UGT1A10, are derived from the same UGT1 gene. Further, the mutation of nucleotide number 1456 at exon 5 causes the mutation of an amino acid at position 486 in the UGT1A1 molecule. This mutation affects the enzymatic activity of UGT1A1, and a mutation in UGT1A6 at the position corresponding to position 1456 of UGT1A1 affects the enzymatic activity of UGT1A6. There are a plurality of reports that describe the relationship between the mutation Y486D of UGT1A1 and enzymatic activity, which were available at the time of filing the present application. As described in page 2 (paragraph 0005) of the present application, for example, a mutation occurs in exon 5 of UGT1A1 gene in Crigler-Najjar syndrome type I and type II and Gilbert syndrome. Due to a mutation (Y486D) in which tyrosine is replaced with aspartic acid at position 486 in the amino acid sequence, the enzyme activity drops to 1/13<sup>th</sup> that of the normal enzyme of UGT1A1.

Meyer et al. (U.S. Patent Application Publication No. 2003/0092019 A1), referenced by the Examiner at page 5 of the Office Action, is inapplicable to the present claims and the teachings of the present application. Meyer et al. states that different mutations in CADPKL genomic sequence (Table 2) are not associated with the same disease. Unlike the present teachings, Meyer et al. does not relate to isoforms of an enzyme. Consequently, the findings in

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Meyer et al. cannot be applied to the present invention.

Also contrary to the Examiner's assertion, the present application does describe the enzymatic reactions for both UGT1A1 and UGT1A6 in glucuronidation of 2-amino-5-nitro-4-trifluoromethylphenol. As stated in pages 7-8 (paragraph 0036) of the present application, the activity of UGT1A1 and UGT1A6 molecules were assayed in glucuronidation of 2-amino-5-nitro-4-trifluoromethylphenol. The results showed that both UGT1A1 mutant and UGT1A6 mutant had less activity than their corresponding wild type molecules (Present application, page 8).

The Examiner points out at page 9 of the Office Action that Kurkela et al. (BIOCHEMICAL PHARMACOLOGY, Vol. 68, 2004, pages 2443-2450) shows that the Y483 mutation in UGT1A9 doubles the Vmax of scopletin glucuronidation, whereas the entacapone glucuronidation rate was decreased. The applicants respectfully point out, however, that the findings in Kurkela et al. are not inconsistent with the teachings of the present application. The present invention provides that by assaying a mutation (Y486D) in the exon 5 region of UGT1A1 and mutations in positions corresponding to (Y486D) in other isoforms of UGT1, it is possible to predict drug metabolism (Present application, page 5). Thus, such a mutation can indicate abnormal drug metabolism. Kurkela et al. shows that a corresponding mutation in UGT1A9 affects the glucuronidation activity of both scopletin and entacapone. The applicants respectfully point out that drug metabolism in a subject is affected whether the mutation causes an increase or a decrease of glucuronidation activity. If the glucuronidation activity decreases, the drugs administered to the subject are insufficiently metabolized. If the glucuronidation activity increases abnormally, the drugs administered to the subject are metabolized excessively. In this case, the drug has less or no effect in the body when the drug is administered in the usual dosage level.

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It should be noted further that the decrease in UGT activity discussed in the present application relates to the activity of a preferred embodiment. Nowhere does the present application state that the changes in UGT1 activity by the mutation are limited to a decrease in UGT activity. In fact, at the priority date of this application, it was known in this technical field that an increase of drug metabolism activity can occur as a result of a mutation in the enzyme. For example, the document, "J PHARMACOL EXP THER. 2001 Dec; 299(3):825-31," attached herewith, states that a mutation in coding region (L293P) of CYP3A4 (isoform of cytochrome P450) can increase the enzymatic activity of CYP3A4 (last two sentences of Abstract). As such, the findings in Kurkela et al. are not inconsistent with the teachings of the present application.

Accordingly, the rejection should be withdrawn.

**Rejection of claims 1-2 and 5-6 under 35 U.S.C. §102(b) -- Huang et al.**

Beginning at page 10 and continuing to page 11 of the Office Action, the Examiner maintains the rejection of claims 1-2 and 5-6 under 35 U.S.C. §102(b), as being anticipated by Huang et al. (PHARMACOGENETICS, Vol. 10, pages 539-44, 2000). The Examiner essentially maintains the rejection presented in the previous Office Action. The rejection is respectfully traversed.

Huang et al. does not teach or suggest the present claims. Unlike the present claims, Huang et al. does not teach or suggest an assay method for drug glucuronidation of UDP-glucuronosyltransferase 1 (UGT1). Claim 1 recites, in part, an assay method for drug glucuronidation of UDP-glucuronosyltransferase 1 (UGT1) of a subject, comprising detecting a mutation(s) in an exon 5 region of a gene coding for UGT1, and determining that, when the

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mutation in an exon 5 region encoding a UGT1 molecule is detected, the level of enzymatic activity of the UGT1 molecule in drug glucuronidation in the subject would be different than that of a UGT1 molecule without the mutation. Claim 1 further specifies that the drug glucuronidation is glucuronidation of 2-amino-5-nitro-4-trifluoromethylphenol.

Huang et al. does not teach or suggest determining that a subject does not have a normal level of enzymatic activity of the UGT1 molecule in glucuronidation of 2-amino-5-nitro-4-trifluoromethylphenol, as presently claimed. In fact, Huang et al. does not describe any relationship between the level of enzyme activity and the mutation of UGT1. Huang et al. describes a method for detecting polymorphism of UGT1A1 using a healthy Taiwanese as a subject, the biochemical assay method (including measurement of bilirubin level) for blood sample of the subject, and the method for examining the relationship of bilirubin level and UGT1A1 (see page 540 of Huang et al.). As such, Huang et al. fails to teach or suggest presently amended claim 1, and the claims dependent thereon.

Accordingly, the applicants respectfully request withdrawal of this rejection.

**Rejection of Claims 10, 13, and 19-21 under 35 U.S.C. §103 (a) – Huang et al. in view of Heinrich et al.**

Beginning at page 11 of the Office Action and continuing to page 13, the Examiner maintains the rejection of claims 10, 13, and 19-21 under 35 U.S.C. §103(a) as being unpatentable over Huang et al., in view of Heinrich et al. (U.S. Publication 2005/0032724 A1). Again, the Examiner essentially maintains the rejection presented in the previous Office Action. This rejection is respectfully traversed.

Huang et al. does not teach or suggest an assay method for drug glucuronidation as recited in claim 1, for the reasons set forth above. As claims 10 and 19 depend from claim 1,

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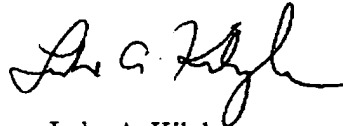
these claims can be distinguished from Huang et al. for the same reasons. Also like claim 1, the methods recited in claims 13 and 20 comprise the step of determining that, when the mutation in an exon 5 region encoding a UGT1 molecule is detected, the level of enzymatic activity of the UGT1 molecule in drug glucuronidation in the subject would be different than that of a UGT1 molecule without the mutation. As discussed above, Huang et al. does not teach determining that a subject does not have a normal level of enzymatic activity of the UGT1 molecule in drug glucuronidation, as presently claimed. Heinrich fails to overcome the deficiencies in Huang et al. Accordingly, withdrawal of the rejection of these claims is respectfully requested.

#### **CONCLUSION**

In view of the foregoing remarks, the applicant respectfully requests the reconsideration of this application and the timely allowance of the pending claims.

If there are any fees due in connection with the filing of this response, please charge the fees to our Deposit Account No. 50-0925. If a fee is required for an extension of time under 37 C.F.R. § 1.136 not accounted for above, such extension is requested and should also be charged to said Deposit Account.

Respectfully submitted,



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Attachment: Article from THE JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS,  
2001 Dec; Vol. 299, No. 3, pages 825-831 (7 pages)



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## Identification of Variants of CYP3A4 and Characterization of Their Abilities to Metabolize Testosterone and Chlorpyrifos

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### ABSTRACT

CYP3A4 is the most abundant isoform of cytochrome P450 (CYP) in adult human liver. It metabolizes numerous clinically, physiologically, and toxicologically important compounds. The expression of CYP3A4 varies 40-fold in individual human livers, and metabolism of CYP3A4 substrates varies at least 10-fold in vivo. Single nucleotide polymorphisms (SNPs) in CYP3A4 were identified by direct sequencing of genomic DNA in 72 individuals from three different ethnic groups, including Caucasians, Blacks (African-Americans and African pygmies), and Asians. A total of 28 SNPs were identified, including five which produced coding changes M445T (CYP3A4\*3), R162Q (CYP3A4\*15), F189S (CYP3A4\*17), L293P (CYP3A4\*18), and P467S (CYP3A4\*19). The latter four represent new allelic variants. Racial variability was observed for the frequency of individual SNPs. CYP3A R162Q was identified only in Black populations

with an allelic frequency of 4%. CYP3A4 F189S and CYP3A4 M445T were identified in Caucasians with allelic frequencies 2% and 4%, respectively. L293P and P467S were only observed in Asians at allelic frequencies of 2%. The cDNAs for the F189S, L293P, M445T, and P467S mutant alleles were constructed by site-directed mutagenesis and expressed in an *Escherichia coli* expression system. Testosterone and the insecticide chlorpyrifos were used to assess the catalytic activities of the most common CYP3A4 allele (CYP3A4\*1) and its allelic variants. CYP3A4 F189S exhibited lower turnover numbers for testosterone and chlorpyrifos, while CYP3A4 L293P had higher turnover numbers for both substrates. The turnover numbers of the CYP3A4 M445T and P467S alleles to metabolize these compounds were not significantly different from those of wild-type CYP3A4.

The CYP3A genes encode the most abundant CYP enzymes in humans including CYP3A4, CYP3A5, CYP3A7, and CYP3A43 (de Wildt et al., 1999; Gellner et al., 2001). Hepatic CYP3A4 has been estimated to metabolize ~50% of currently used drugs as well as a number of steroids, environmental chemicals, and carcinogens (Aoyama et al., 1989; Shimada et al., 1994; Thummel et al., 1996; Rebbeck et al., 1998; Guengerich, 1999). CYP3A4 is considered to be the predominant form in adult human liver. CYP3A5, a polymorphic form, is present to a variable extent in adult livers. CYP3A5 is believed to be present in the livers of approximately 20% of Caucasians, but a recent study suggests that CYP3A5 is

expressed and may predominate in more than 50% of African-Americans (Lown et al., 1994; de Wildt et al., 1999; Wandel et al., 2000; Kuehl et al., 2001). Both CYP3A4 and CYP3A5 are distributed in multiple tissues including not only liver, but also intestine and kidney (Thummel and Wilkinson, 1998; Guengerich, 1999). CYP3A7 is an isoform found in intestine, reproductive organs, and infant liver but is also present in some adult livers (Kitada et al., 1985; Schuetz et al., 1994). Recently, a new CYP3A member (CYP3A43) has been identified. CYP3A43 mRNA is found predominantly in adult prostate and is also present in multiple tissues, including liver, where it is inducible by rifampicin (Gellner et al., 2001). However, Westlind et al. (2001) using heterologous expression systems including yeast, COS-1 cells, mouse hepatic H2.35 cells, and human embryonic kidney 293 cells suggested that CYP3A43 was a non-functional isoform.

CYP3A levels fluctuate in the liver throughout the life span of an individual (Shimada et al., 1994; Oesterheld,

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**ABBREVIATIONS:** CYP, cytochrome P450; SNP, single nucleotide polymorphism; OPs, organophosphorus; TLC, thin layer chromatography; TCP, trichloropyridinol; CPO, chlorpyrifos-oxon; PCR, polymerase chain reaction; HPLC, high-pressure liquid chromatography; NADPH,  $\beta$ -nicotinamide adenine dinucleotide phosphate, reduced form; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid.

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1998). Up to 40-fold interindividual variations in expression levels of CYP3A4 have been observed in human liver. There is an approximately 10-fold variation in metabolism of CYP3A4 substrates in vivo including the antibiotics rifampicin and ketoconazole, the calcium blocker nifedipine, and the immunosuppressant cyclosporine (Thummel and Wilkinson, 1998; Guengerich, 1999). This variation can affect drug efficacy and toxicity. CYP3A4 is inducible by drugs such as rifampicin (Kolars et al., 1992). The variable expression of CYP3A4 is at least partially due to multiple factors, including induction by drugs, endogenous compounds, and environmental chemicals, but also includes genetic factors. Recent evidence suggests that the coding region of CYP3A4 is also genetically variable (Sata et al., 2000; Eiselt et al., 2001).

CYP3A4 has also been shown to be important in the metabolism of organophosphate pesticides (OPs), such as chlorpyrifos (Tang et al., 2001) and parathion (Butler and Murray, 1997; Eaton, 2000). Chlorpyrifos is a widely used broad-spectrum OP insecticide that elicits toxicity through inhibition of acetylcholinesterase (Chambers, 1992). OPs inhibit acetylcholinesterase and exert their toxicity by causing the accumulation of the neurotransmitter acetylcholine at nerve synapses and neuromuscular junctions. These OPs are used as the phosphorothioate ( $P = S$ ), which is a very weak inhibitor of acetylcholinesterase. However, OPs are converted in vivo from ( $P = S$ ) to an active phosphate ester or oxon ( $P = O$ ), which is a potent acetylcholinesterase inhibitor (Chambers, 1992) by CYP enzymes.

Genetic variations of CYP3A4 have recently been reported. A mutation in the 5'-upstream region termed *CYP3A4\*1B* (A290G) was observed in 52% of African-Americans and 9.6% of Caucasians, but has not been identified in Asians (Ball et al., 1999; Rebbeck, 2000; Sata et al., 2000; Gellner et al., 2001). It was suggested to be associated with advanced stage prostate cancer in men (Rebbeck et al., 1998), yet has protective effects for secondary cancer caused by chemotherapeutic drugs for leukemia metabolized by CYP3A4, such as epipodophyllotoxins (Felix et al., 1998). However, this polymorphism does not appear to affect constitutive levels of CYP3A4 (Wandel et al., 2000). Gonzales and coworkers (Sata et al., 2000) have described two coding SNPs including *CYP3A4\*2* (S222P) found only in Finnish Caucasians with an allelic frequency of 2.7%, and a single case of *CYP3A4\*3* (M445T) in a Chinese population of 178 individuals. Baculovirus expressed CYP3A4\*2 protein exhibited an increase in the  $K_m$  for nifedipine but not for testosterone compared with CYP3A4\*1. There has been limited information about the effects of a new M445T allele on metabolism (Sata et al., 2000). *CYP3A4\*4* (I118V), *CYP3A4\*5* (P218R), and *CYP3A4\*6* (a stop codon at 285) were reported in a Chinese population with allelic frequencies of 1.4%, 0.98%, and 0.5%, and all of these variant alleles were associated with lower ratios of 6 $\beta$ -hydroxycortisol to free cortisol in an in vivo study (Hsieh et al., 2001). A very recent study has identified seven new polymorphisms in European Caucasians (Eiselt et al., 2001).

To identify CYP3A4 polymorphisms, we screened for single nucleotide polymorphisms among 72 individuals from three different racial groups including ethnically diverse Caucasians, Blacks (African-Americans and African pygmies) and ethnically diverse Asians. We identified four new coding polymorphisms in CYP3A4. A bacterial cDNA expression system was used. Catalytic activities of wild-type CYP3A4

and allelic variants were compared using testosterone and the insecticide chlorpyrifos as prototypic 3A4 substrates.

## Materials and Methods

Testosterone,  $\beta$ -nicotinamide adenine dinucleotide phosphate, reduced form (NADPH), isopropyl  $\beta$ -D-thiogalactopyranoside,  $\delta$ -aminolevulinic acid, phenylmethylsulfonyl fluoride, phosphatidylcholine, and leupeptin were purchased from Sigma Chemical Co. (St. Louis, MO).  $^{14}$ C-Testosterone was purchased from Invitrogen (Boston, MA). Chlorpyrifos, chlorpyrifos-oxon (CPO), and 3,5,6-trichloro-2-pyridinol (TCP) were purchased from ChemService (West Chester, PA). HPLC grade acetonitrile and methanol were purchased from Fisher Scientific (Fair Lawn, NJ). Human NADPH reductase was obtained from Oxford Biomedical Research (Oxford, MI). All restriction enzymes were obtained from New England Biolabs (Beverly, MA). Taq polymerase was purchased from QIAGEN (Valencia, CA). Anti-CYP3A4 antibody was obtained from GENTEST (Woburn, MA).

**Direct Sequencing.** Genomic DNA was obtained from 72 different human lymphoblastoid cell lines (Cornell Institute, Camden, NJ). The individuals contain the following varied racial and ethnic ancestries: 24 Africans (16 African-Americans and 8 African pygmies), 24 Asians (5 native Taiwanese, 5 mainland Chinese, 4 Melanesian, 4 Indo-Pakistani, 3 Cambodian, and 3 Japanese), and 24 Caucasians (9 from Utah, 5 Druze [Lebanon], 5 eastern European, and 5 from Moscow).

**Variant Identification.** The exons plus splice junctions and the 5' and 3' regions of CYP3A4 were sequenced as previously described by Shen et al. (1998). Briefly, PCR primers were located so that amplification of the genomic sequence is initiated approximately 50 nucleotides from each intron-exon boundary. This is sufficient distance for high quality sequence data to be obtained before reaching the intron/exon splice site. Appended to the 5'-end of each of the PCR primers were sequences containing the primer binding sites for the forward or reverse energy transfer DNA sequencing primers (Amersham Pharmacia Biotech, Cleveland, OH). The amplification products are directly sequenced according to the manufacturer's instructions using the DYEnamic Direct cycle sequencing kit with the DYEnamic energy transfer primers (Amersham Pharmacia Biotech). The denatured products are loaded onto ABI Prism 377 stretch DNA sequencers (Foster City, CA). "PolyPhred" (version 2.1), a software package that utilizes the output from Phred, Phrap, and Consed, was used to identify single nucleotide substitutions in heterozygous individuals (Nickerson et al., 1997; Rieder et al., 1998). A nucleotide sequence analysis program (<http://genomic.sanger.ac.uk/gf/gftl.html>) was used to predict possible new splice sites introduced by any new mutations.

**Modification of CYP3A4 cDNA.** CYP3A4 wild-type cDNA in the vector pUC19 was generously supplied by Frank Gonzales (National Cancer Institute, National Institute of Health). N-Terminal modification of CYP3A4 cDNA included removal of the initial 10 amino acids and conversion of the first eight amino acids of CYP3A4 into those of bovine 17 $\alpha$ -hydroxylase (MALLAVF). This was accomplished by PCR using sense primer: 5'-TTAGGAGGTCATATGCTCTGTTATTAGCAGTTTTTCTGGTGCTCCTCTAT-3', which introduced a unique restriction site for *NdeI*. The antisense primer (5'-AGCAGAAGTCTCTAGAAAAATTCAGGCTCCACTTACGGTGC-3') was used to introduce an *EcoRI* site. *NdeI* and *EcoRI* sites are unique for the expression vector pCW. Amplification of CYP3A4 ORF was accomplished by PCR with Pfu polymerase using primers described above. PCR products containing an open reading frame of CYP3A4 were digested by *NdeI* and *EcoRI* and then were subcloned into pCW. Fidelity of PCR was verified by complete sequencing of CYP3A4. Sequentially, the plasmids were transformed into *E. coli* XL1 Blue cells.

**Site-Directed Mutagenesis.** Five mutations containing R162Q, F189S, L293P, M445T, and P467S were made using site-directed mutagenesis. A Chameleon double-stranded site-directed mutagen-

esis kit from Stratagene (La Jolla, CA) was used to introduce single nucleotide changes (indicated in lower case and boldface): primer 5'-GGTGAGAAATCTGAGGCaGGAAGCAGAGACAGG-3', was used to produce the substitution R162Q. The primer 5'-GTGATCACTAG-CACATCATcTGGAGTGAACATCGACTC-3' was used to substitute individual nucleotide changes coding for the F189S substitution in exon 7. Primer 5'-CAAAGCTCTGTCCGATCcGGAGCTCGTG-GCCCAATC-3' introduced the substitution L293P in exon 10. Primer 5'-CTGCATTGGCAcGAGGTTTGCTCTC-3' introduced M445T in exon 12. Primer 5'-CAGAACTTCTCCTTCAAAtCTTGTA-AAGAAACACAGATCCC-3' introduces P467S in exon 12. The entire coding region, including the mutated sites, was verified by sequencing with an ABI PRISM 377 DNA sequencer (PerkinElmer Life Sciences, Foster City, CA). The entire cDNA was then excised and subcloned into a new pCW plasmid to avoid any accidental mutations in the plasmid caused by the mutagenesis procedure and expressed in *E. coli* XL1 Blue.

**Expression and Partial Purification of CYP3A4s.** Wild-type and variant CYP3A4 alleles were expressed in *E. coli* XL1 Blue and the allelic proteins were purified as previously described (Dai et al., 2001). Cytochrome P450 content was monitored by the reduced CO spectrum using a DW-2000 Spectrophotometer. Protein concentration was determined by the method of Lowry (Dawson and Heatlie, 1984).

**Western Blot Analysis.** SDS-polyacrylamide gel electrophoresis was used to separate the recombinant proteins, followed by transferring the proteins onto nitrocellulose membranes. Nonspecific binding was blocked by 10% nonfat milk for 1 h. The membranes were incubated with anti-CYP3A4 primary antibody for 1 h at room temperature. An enhanced chemiluminescent kit (Pierce, Rockford, IL) was used for immunodetection.

**Testosterone Metabolism.** Metabolism of testosterone by the recombinant wild-type and mutant CYP3A4 alleles was characterized. The purified recombinant CYP3A4 proteins (10 pmol) were reconstituted in with 0.4% CHAPS, 1  $\mu$ g of dioleoylphosphatidylcholine, and 40 pmol of human NADPH reductase (Oxford Biomedical Research, Oxford MI) and 20 pmol of cytochrome  $b_5$  in 1 $\times$  HEPES buffer, pH 7.6 (50 mM HEPES, 15 mM MgCl<sub>2</sub>, and 0.1 mM EDTA) a 10- $\mu$ l volume. The reconstitution mixture was preincubated at 37°C for 5 min and then diluted to a final volume of 100  $\mu$ l with 1 $\times$  HEPES containing 10  $\mu$ g of dioleoylphosphatidylcholine. The optimal conditions for this substrate were generously provided by Drs. Halpert and He at the University of Texas Medical School in Galveston Texas. The reaction mixture was preincubated at 37°C for 5 min, and the reaction initiated by addition of 10  $\mu$ l of 10 mM NADPH and terminated with 50  $\mu$ l of tetrahydrofuran. All incubations were performed in triplicate. Samples were analyzed by thin layer chromatography (TLC) using a solvent system of dichloromethane/acetone (4:1, v/v). Finally, the TLC plate was exposed to radioautography and analyzed. Turnover numbers for CYP3A4\*1 and mutants were determined by counting the radioactivity of the TLC spots.

**Chlorpyrifos Metabolism.** CYP3A4s (100 pmol) were reconstituted with dioleoylphosphatidylcholine (3  $\mu$ g/10 pmol P450), NADPH reductase (400 pmol), and cytochrome  $b_5$  (200 pmol) added in this order. The reaction was initiated by adding 100  $\mu$ M chlorpyrifos in 100 mM potassium phosphate buffer with 3.3 mM MgCl<sub>2</sub> (pH 7.4) with the NADPH generating system (the final concentration was 0.25 mM NADP, 2.5 mM glucose 6-phosphate, and 2 U/ml glucose-6-phosphate dehydrogenase). The final assay volume was 500  $\mu$ l. The 30-min incubation was terminated by the addition of 500  $\mu$ l of ice-cold acetonitrile and vortexing. After 5 min of centrifugation at 15,000 rpm, the supernatant was analyzed for chlorpyrifos-oxon and trichloropyridinol concentrations by HPLC. The HPLC system used in this study consisted of two Shimadzu pumps (LC-10AT; Kyoto, Japan) and a Shimadzu auto injector (SIL-10AD VP). The mobile phase for pump A was 10% acetonitrile, 89% water, and 1% phosphoric acid, whereas for pump B it was 99% acetonitrile and 1% phosphoric acid. A gradient system was initiated at 20% pump B and increased to 100% pump B in 20 min. The flow rate was 1 ml/min.

Metabolites were separated by a C<sub>18</sub> column (Synergi Max 4  $\mu$ , 150  $\times$  4.6 mm, Phenomenex, Rancho Palos Verdes, CA) and detected at 230 nm by a Waters 486 tunable absorbance detector (Milford, MA). Concentrations of metabolites were obtained by extrapolation of peak height from a standard curve.

**Statistical Analysis.** All enzymatic data were analyzed by analysis of variance followed by Student's *t* test. *N* is the number of samples used in study. Differences were considered significant at *P* < 0.05.

**Molecular Modeling.** A molecular model was developed for the human CYP 3A4 wild-type protein using the technique of comparative/homology modeling. The polymorphism residue side chains were identified and modified in the completed wild-type model. The template structure used for development of the homology model was the solved mammalian microsomal rabbit cytochrome P450 2C5/2C3 chimeric structure (protein database entry: 1DT6) (Williams et al., 2000). The Molecular Simulations homology modeling package was used in a manual mode for development of the homology model. All molecular dynamics studies of the protein were performed using the Discover, Lifson and Hagler, Consistent Valence Force Field. The model was developed based on a multiple sequence alignment (Fig. 4) of the solved crystal structure (protein database: 1DT6) sequence with human cytochrome P450 2C8, 2C9, 2C18, 2C19, and 3A4 sequences. The multiple sequence alignment was performed manually based on the published P450 alignments of Gotoh (1992; Lewis, 1998) and D. Nelson (<http://drnelson.utmem.edu>). The model required insertion of seven small loops ranging from three to nine residues inserted using the loop generation program present within the Molecular Simulations homology program. There were no deletions. Discontinuities, steric bumps, and overlaps were resolved with molecular dynamics.

## Results

**Direct Sequencing.** Genomic sequencing of all exons and intron-exon junctions was performed on DNA from 72 different human lymphoblastoid cell lines selected from individuals of varied racial and ethnic ancestries [24 individuals with African ancestry (16 African-Americans and 8 African pygmies), 24 Asians (5 Indo-Pakistani, 5 native Taiwanese, 5 mainland Chinese, 3 Cambodians, 3 Japanese, 3 Melanesian) and 24 Caucasians (10 from Utah in the United States, 5 Druze (Lebanon), 5 eastern Europeans, and 5 Russians)]. Twenty-eight SNPs were identified in these regions of CYP3A4 (Table 1). Eight SNPs were located in the exonic regions: R162Q, F189S, I193I, L293P, A297A, T346T, M445T, and P467S. The remaining SNPs were distributed in the 5'-upstream, introns, and 3'-flanking region. Sequencing results showed that R162Q was only detected in Black populations with an allelic frequency of 4% (African-Americans 7.1%, pygmies 0%). F189S was detected only in Caucasians with an allelic frequency of 2% (ethnic frequencies 10% in Eastern Europeans, not found in other Caucasian groups). Two SNPs were only found in Asians. L293 was found in Asians with a frequency of 2% (ethnic frequencies of 10% in Chinese, 0% in other Asian groups). P467S was also found in Asians with an allelic frequency of 2% (ethnic frequencies were 12% in Indo-Pakistani and 0% in other Asian ethnic groups). M445T was only detected in Caucasians in our study with an allelic frequency of 4% (Eastern Europeans: frequency of 10%, Caucasians from Utah 5.6%, not detected in other Caucasian ethnic groups). None of the samples was homozygous for the coding SNPs. No new putative splice sites were introduced by any of these coding, noncoding, or intron SNPs.

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TABLE 1  
CYP3A4 SNPs<sup>a</sup>

SNPs	Site	Amino Acid Substitution	Nucleotide Substitution	Allelic Frequencies			Location in CYP3A4 Genome
				Africans 24	Caucasians 24	Asians 24	
				%			
1	5'-UTR		aatcc(A/G)acncc	0	0	2.1	148874
2	Intron 2		ttcca(T/C)tggt	14.6	0	0	154915
3	Intron 3		agctc(T/A)gtca	0	0	2.1	155157
4	Intron 4		aactg(A/T)gttag	0	0	2.1	162802
6	Intron 5		tggtg(T/G)gtgt	0	0	2.1	163198
6	Intron 6		ccagc(T/G)gctg	0	4.2	0	163355
7	Exon 6	R162Q	gaggg(G/A)ggag	4.2	0	0	163267
8	Intron 7		atctt(T/G)ctctc	50.0	2.1	2.2	164751
9	Intron 7		tgaga(T/C)ataaa	8.7	0	0	164781
10	Intron 7		attca(T/G)ccact	2.2	0	0	164802
11	Intron 7		tgtag(T/C)acatt	6.5	0	0	164835
12	Exon 7	F189S	atcat(T/C)tgag	0	2.1	0	164613
13	Exon 7	Silent	aacat(C/T)gactc	4.2	0	0	164626
14	Intron 9		gacac(AT/-)gtttg <sup>b</sup>	0	0	4.2	166813
15	Intron 10		ggatg(G/A)taaat	73	14.6	37.5	169228
16	Intron 10		cttag(C/T)aaaaa	8.3	0	0	169263
17	Intron 10		aaaaa(G/C)ataaa	10.4	0	0	169307
18	Intron 10		gtttc(G/A)ttctt	2.1	0	0	170793
19	Exon 10	L293P	cgatc(T/C)ggagc	0	0	2.1	169068
20	Exon 10	Silent	gtggc(C/T)caate	2.1	0	0	169081
21	Intron 11		aagaa(A/G)cccta	0	2.1	0	172022
22	Intron 11		accaa(C/T)gttga	21	0	2.1	172079
23	Exon 11	Silent	ccca(c/G)tatga	0	0	2.1	170821
24	Exon 12	M445T	tggca(T/C)gaggt	0	4.2	0	172170
25	Exon 12	P467S	tcaaa(C/T)cttgt	0	0	2.1	172235
26	3'-UTR		aaata(A/T)ccggg	4.2	0	0	175065
27	3'-UTR		gtaca(T/G)gcatt	2.1	0	0	175082
28	3'-UTR		ctgca(C/T)attaa	2.1	0	0	175416

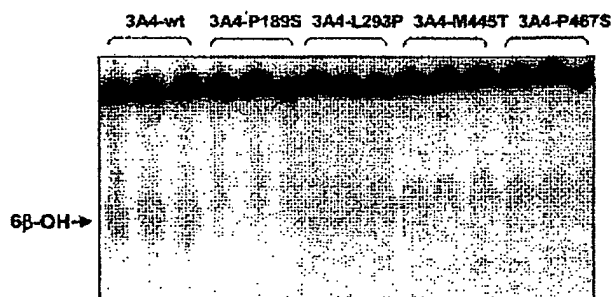
UTR, untranslated region.

<sup>a</sup> Accession number of CYP3A4 genomic DNA in GenBank is NG\_000004.<sup>b</sup> Deletion 2 bases (AT).

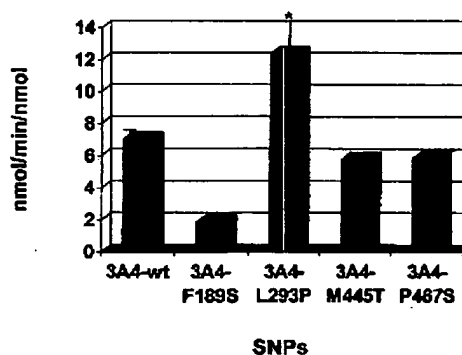
**Expression and Enzymatic Assay of CYP3A4 Recombinant Alleles.** The CYP3A4 alleles were inserted into the pCW expression vector, expressed in *E. coli*, and partially purified with the exception of the newly discovered R162Q allele, which we are currently attempting to express. Comparison of Western blotting (data not shown) and CO spectra of the mutants indicated that all were present as the holoprotein. Both CYP3A4\*1 and all mutant CYP3A4s metabolized radioactive testosterone into 6 $\beta$ -OH testosterone as the only detectable metabolite (Fig. 1). CYP3A4-P189S exhibited a lower turnover number (1.9 nmol/min/nmol) than CYP3A4\*1 (7.03 nmol/min/nmol) ( $P < 0.05$ ). Conversely, CYP3A4-L293P metabolized testosterone at a higher rate

(12.4 nmol/min/nmol) than CYP3A4\*1 ( $P < 0.05$ ) (Fig. 2). The turnover numbers for M445T and P457S were 5.8 and 5.9 nmol/min/nmol, respectively.

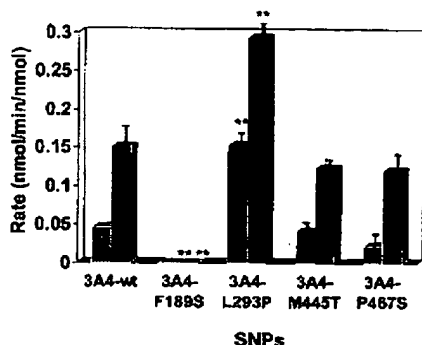
**Chlorpyrifos Metabolism.** The active metabolite chlorpyrifos-oxon and the inactive product trichloropyridinol were the major metabolites of chlorpyrifos by CYP3A4 (Figs. 3 and 4). The mutant allele L293P exhibited an increased turnover number for both metabolites ( $P < 0.01$ ). In contrast, the F189S allele exhibited a lower turnover number for formation of both CPO



**Fig. 1.** Metabolism of testosterone by CYP3A4s. TLC profile of testosterone hydroxylation assay. Purified recombinant CYP3A4 wild-type and mutant proteins (10 pmol) were reconstituted and incubated with <sup>14</sup>C-testosterone (25  $\mu$ M) for 5 min as described under *Materials and Methods*. Each assay was run in triplicate. 6 $\beta$ -OH testosterone was the only metabolite detected in significant amounts.



**Fig. 2.** Metabolism of testosterone. 6 $\beta$ -OH-testosterone is the principle metabolite produced by CYP3A4\*1 and its allelic variants. Purified recombinant CYP3A4 wild-type and mutant proteins (10 pmol) were reconstituted and incubated with testosterone (25  $\mu$ M) for 5 min as described under *Materials and Methods*. The turnover numbers for CYP3A4\*1, F189S, L293P, M445T, and P467S are 7.03, 1.9 ( $P < 0.05$ ), 12.4 ( $P < 0.05$ ), 5.8, and 5.9 nmol/min/nmol, respectively.



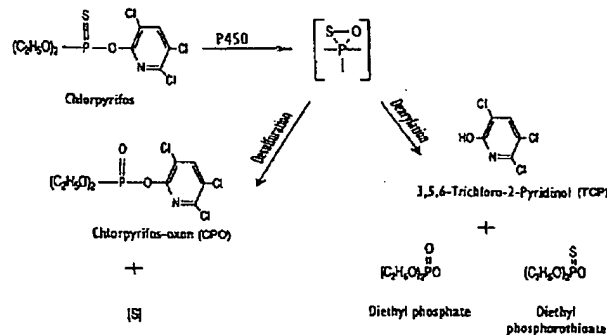
**Fig. 3.** Metabolism of chlorpyrifos CYP3A4 metabolizes chlorpyrifos into TCP (□) and CPO (■). CYP3A4s (100 pmol) were reconstituted and were then incubated with 100  $\mu$ M chlorpyrifos in 100 mM potassium phosphate buffer with 3.3 mM  $MgCl_2$  (pH 7.4) with the NADPH generating system (the final concentration was 0.25 mM NADP, 2.5 mM glucose 6-phosphate, and 2 U/ml glucose-6-phosphate dehydrogenase) for 30 min. The metabolites of chlorpyrifos-oxon (CPO) and trichloropyridinol (TCP) were analyzed by HPLC.

and TCP. The remaining two alleles did not significantly change the turnover numbers for either metabolite.

**Model of CYP3A4** CYP3A4 was aligned with rabbit CYP2C5. Figure 5 shows the results of modeling CYP3A4 based on the known crystal structure of CYP2C5 (protein database entry: 1DT6). Mutations are indicated in blue. The heme is indicated in purple and the substrate testosterone in gray. The identified variants are predicted from the model to be located near the following identified structural features; R162Q is located at the end of helix D, F189S at the end of helix E, L293P is at the beginning of helix I, M445T at the beginning at helix L and P467S in a  $\beta$ -sheet near the C terminus of the protein. The M445T SNP is located on the other side of the heme from the ligand binding site and helix I. Side chains of residues F189, L292, and P467 are largely buried and packed into the interior of the protein. Side chains of residues R162 and M445 are largely surface exposed.

## Discussion

CYP3A4 is known to metabolize many clinically important drugs, such as rifampicin, cyclosporine, and ritonavir (Kolars et al., 1992; Boxenbaum, 1999; Guengerich, 1999; Hesse et al., 2001) as well as endogenous compounds such as testosterone (Wang et al., 1997). The distribution of metabolism of CYP3A substrates is unimodal but metabolism of these sub-



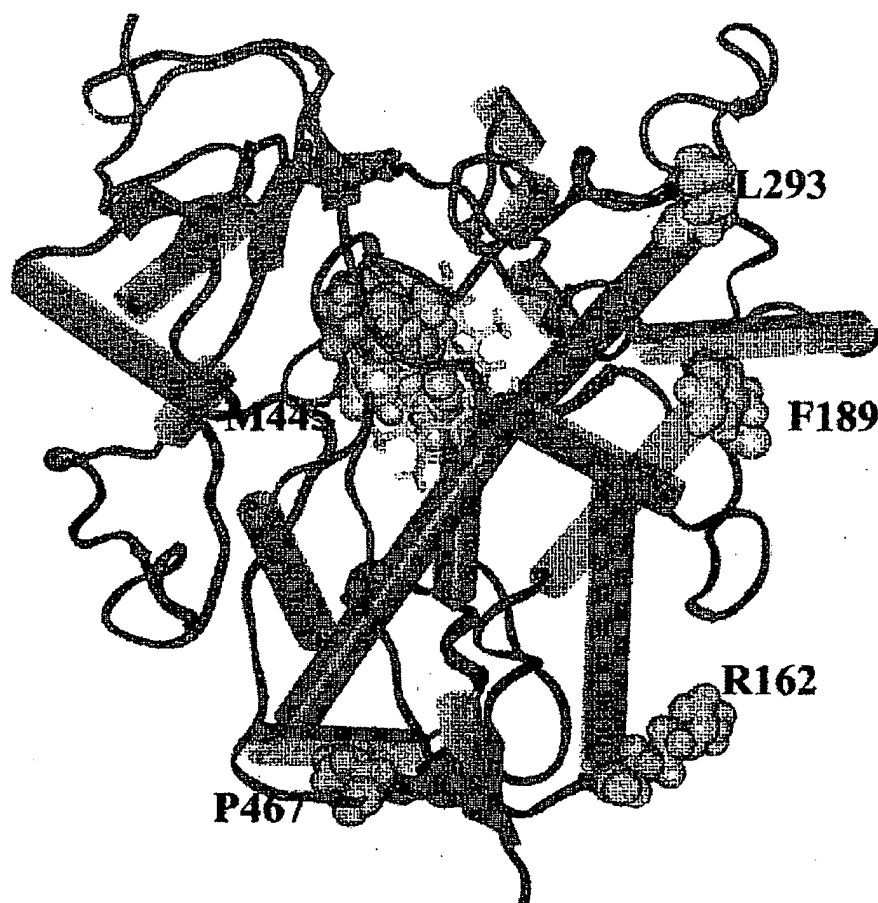
**Fig. 4.** Metabolic pathway of chlorpyrifos by P450.

strates shows at least a 10-fold variability in vivo (Thummel et al., 1996). Some variability may be due to the inducibility of the CYP3A4 gene by drugs and environmental chemicals. However, some of this variability is believed to be due to genetic factors. A previous study found a S222P allele in Finnish Caucasians and a M445T allele in Chinese (Sata et al., 2000). A very recent study identified seven new polymorphisms in European Caucasians (Eiselt et al., 2001). Two of these alleles, S222P and L373F, have been reported to affect catalytic activities toward certain substrates (Sata et al., 2000; Eiselt et al., 2001).

In the present study, we sequenced the coding regions and intron-exon junctions of CYP3A4 in DNAs from three different racial groups. Each racial group had been selected to represent ethnic diversity. A total of 28 SNPs in CYP3A4 were detected. Five SNPs produced amino acid substitutions including R162Q in exon 6; F189S in exon 7; L293P in exon 10; M445T in exon 12; and P467S in exon 12. Four of these are newly described alleles. Only the M445T allele had previously been reported (Sata et al., 2000; Eiselt et al., 2001). P189S and M445T were detected only in Caucasians in our study with frequencies of 2% and 4%, respectively. However, M445T has also been reported in Asians (Sata et al., 2000), indicating it is of ancient ancestry. The L293P and P467S alleles were found only in Asians, both at frequencies of 2% (L293 occurred in Chinese with a frequency of 10% while P467S occurred in Indo-Pakistani with a frequency of 12.5%). The coding change R162Q occurred only in African-Americans. In both individuals, this SNP was associated with an SNP in intron 10 (bp 169228) of the gene, in intron 7 (bp 164751), and in intron 11 (bp 172079). However, these intron SNPs were more frequent than the R162Q SNP in Africans. Interestingly, all alleles in African pygmies, and the majority (19/28) of alleles in African-Americans carried the SNP in intron 10 (bp 169228), which was not frequent in Caucasians. This SNP was also frequent in Asians (37.5%), indicating that it is associated with an ancient allele. The intron 7 SNP was also frequent in Africans (50% of the samples).

Testosterone and the OP insecticide chlorpyrifos were used as two examples of substrates for CYP3A4 to test effects of the coding mutations on function. The CYP3A4 active site is large (He et al., 1997), which explains its ability to metabolize a wide group of structurally diverse pharmacores (Ekins et al., 1999). Testosterone was selected as an example of a spatially large molecule that is metabolized by CYP3A4. Coding polymorphisms might potentially affect orientation of large substrates preferentially over their effects on binding of smaller substrates. Interestingly, the F189S and L293P mutations affected metabolism of both substrates in a similar fashion. The F189S allele exhibited significantly lower turnover numbers for both testosterone and chlorpyrifos than wild-type CYP3A4, while the L293P allele exhibited higher turnover numbers for both substrates. These results indicated that individuals might potentially have alterations in their ability to metabolize not only testosterone but potentially other pharmacores. Future studies will address metabolism of clinically important drugs.

Based on comparisons of the model of CYP3A4 with the crystal structure of CYP2C5, we would predict that the mutation at L293P is at the beginning of the I helix while residue F189S is at the end of helix E. These residues are not predicted to reside in the active-site cavity where they would



**Fig. 5.** Crystal structure of CYP3A4 showing locations of amino acid variants. Ribbon diagram of the molecular model of the human cytochrome P450 3A4 based on the solved x-ray crystal structure of the rabbit microsomal cytochrome P450 2C5 (protein database entry: 1DT8) (Williams et al., 2000). Complete side chains are shown in blue for the identified SNPs. The substrate testosterone is shown in gray docked in the enzyme active site. The heme is shown in purple.

directly interact with the substrate. However, the residues are nonconservative mutations in tightly packed regions, which could conceivably affect the conformation of the protein, substrate access, and/or catalytic activity. Our results indicating that the M445T mutation has no effect on testosterone or chlorpyrifos metabolism are consistent with the very recent report by Eiselt et al. (2001) using testosterone and progesterone as substrates. Modeling based on the crystal structure of CYP2C5 predicts that the M445T SNP is located in close proximity to the heme, but it is on the opposite side of the heme from the ligand binding site on helix I. This is consistent with the data indicating that this mutation may not affect catalytic activity.

CYP3A4 is important in the metabolism of environmental compounds as well as clinically important drugs. CYP3A4 is known to activate the OP insecticides parathion and chlorpyrifos into oxons that are neurotoxicants (Butler and Murray, 1997; Tang et al., 2001) (Fig. 5). CYP3A4 also inactivates chlorpyrifos into 2,3,5-trichloro-2-pyridinol. The relative rates of activation and inactivation are critical to the toxicity of the compound. The L293P allele could possibly increase

the toxicity of OP insecticides to individuals carrying this allele. Interestingly, the F189S allele decreased both activation and inactivation of chlorpyrifos and could also potentially affect toxicity after exposure to OP insecticides. In addition, CYP3A4 can activate aflatoxin B<sub>1</sub> into the reactive form, aflatoxin B<sub>1</sub>-8, 9-epoxide, which is a mutagen (Gallagher et al., 1996; Chen et al., 1998). Thus polymorphisms of CYP3A4 could potentially influence the risk of different populations from various environmental compounds.

In summary, five coding polymorphisms in CYP3A4 were identified as M445T (CYP3A4\*3), R162Q (CYP3A4\*15), F189S (CYP3A4\*16), L293P (CYP3A4\*17), and P467S (CYP3A4\*18) by resequencing 72 individuals from three diverse racial groups.<sup>1</sup> Four of these represent newly described CYP3A4 alleles. Two SNPs occurred in Caucasians (F189S

<sup>1</sup>New CYP3A4 alleles were submitted to the CYP allele web page ([www.imm.ki.se/CYPalleles](http://www.imm.ki.se/CYPalleles)). The names designated by the international allele nomenclature committee are CYP3A4\*17 (F189S) and CYP3A4\*18 (L292P and CYP23A4\*19 (P467S). R162Q was submitted to the CYP3A4 allele web page by another laboratory while this work was in progress and was designated CYP3A4\*15 but has not yet been published otherwise.

and M445T), while two occurred in Asians (L293P and P467S). M445T has also been reported previously in Asians. One coding SNP R162Q was detected only in African-Americans. Testosterone and the OP insecticide chlorpyrifos were selected to assess their catalytic activities of four new alleles. The F189L allele exhibited significantly a lower turnover number for both substrates than CYP3A4\*1, while the L293P allele metabolized both substrates with a higher turnover number. Potentially, these alleles may contribute to the known variability in metabolism of clinically used drugs and environmental compounds that are CYP3A4 substrates. Future studies will examine a wider range of CYP3A4 substrates including clinically used drugs.

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